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(54) **Process for producing L-threonine and L-isoleucine.**

(57) L-threonine and L-isoleucine are produced by constructing a recombinant DNA containing a gene coding for both enzymes of homoserine dehydrogenase and homoserine kinase responsible for the threonine biosynthesis in a microorganism belonging to the genus *Corynebacterium* or *Brevibacterium*, incorporating the recombinant DNA in a microorganism belonging to the genus *Corynebacterium* or *Brevibacterium*, culturing the microorganism in a medium, and recovering L-threonine or L-isoleucine accumulated in the culture broth.

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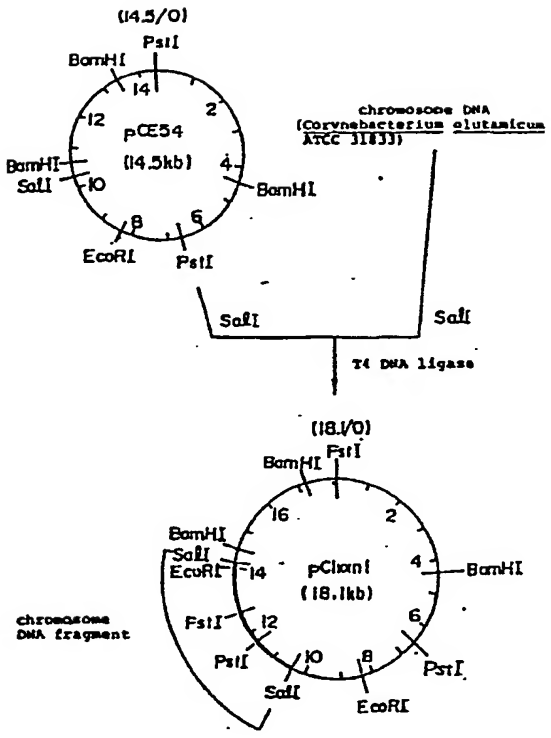
Fig 1.


(14.5/0)
PstI
BamHI
14
2
12
pCE54
(14.5kb)
4
BamHI
BamHI
SalI
10
8
6
EcoRI
PstI
chromosome DNA
(Corynebacterium glutamicum
ATCC 31833)
SalI
SalI
T4 DNA ligase
(18.1/0)
PstI
BamHI
16
2
BamHI
SalI
EcoRI
14
pClxan1
(18.1kb)
4
BamHI
6
PstI
12
PstI
10
8
PstI
SalI
EcoRI
chromosome
DNA fragment

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TITLE OF THE INVENTION

PROCESS FOR PRODUCING L-THREONINE AND L-ISOLEUCINE

Background of the Invention

As for the processes for producing L-threonine and L-isoleucine by fermentation using microorganisms belonging to the genus Conynebacterium or Brevibacterium, processes using mutants derived from wild-type strains of the said bacteria are known. As the mutant strains producing L-threonine and L-isoleucine, strains having an amino acid-requiring mutation and/or an amino acid analog-resistant mutation are known, as disclosed, for example, in Japanese Unexamined Published Patent Application No. 19087/72 or Japanese Patent Publication No. 32070/79, etc. Besides the processes using the mutant strains, processes using strains established by a recombinant DNA technique are known. For example, there are disclosed processes for producing L-threonine or L-isoleucine by fermentation using a strain belonging to the genus Corynebacterium or Brevibacterium and carrying a recombinant plasmid DNA which contains a DNA fragment carrying the genetic information of an enzyme responsible for the threonine biosynthesis in Escherichia coli (Japanese Unexamined Published Patent Application Nos. 126789/83 and 30693/85).

Furthermore, there is disclosed a process for producing L-threonine or L-isoleucine by the strains belonging to the genus Corynebacterium or Brevibacterium and carrying a recombinant plasmid DNA which contains a gene coding for homoserine dehydrogenase (referrred to as HD hereinafter) of the genus Brevibacterium (referred to as HD gene hereinafter) (Japanese Unexamined Published Patent Application No. 12995/85).

With the recent increase in the demand for L-threonine and L-isoleucine, an improvement in the processes

for producing these amino acids has been desired. To meet this problem, the present inventors have made extensive studies for increasing the ability of the strains of the genus Corynebacterium or Brevibacterium to produce L-threonine and L-isoleucine by a recombinant DNA technique.

The present inventors have found that the ability of a strain of the genus Corynebacterium or Brevibacterium to produce L-threonine and L-isoleucine to be biosynthesized from L-threonine as a precursor can be considerably improved by introducing into the strain a recombinant plasmid DNA containing genetic information of HD and homoserine kinase (referred to as HK hereinafter) at the same time, among the enzymes responsible for the biosynthesis of L-threonine in a microorganism belonging to the genus Corynebacterium or Brevibacterium and have established the present invention. As for L-threonine-producing strains or the L-isoleucine-producing strains carrying a recombinant plasmid DNA containing a gene originating from a strain of the genus Corynebacterium or Brevibacterium, a strain containing HD gene has been disclosed, as mentioned before, but there have been no examples of strains containing both genes, HD gene and a gene coding for HK (referred to as HK gene hereinafter), and it has been found in the present invention for the first time that the recombinant DNA containing both genes can remarkably contribute to the ability to produce L-threonine and L-isoleucine.

Summary of the Invention

The present invention relates to a process for producing L-threonine or L-isoleucine, which comprises constructing a recombinant DNA containing a gene coding for both enzymes of HD and HK responsible for the threonine biosynthesis in a microorganism belonging to the genus Corynebacterium or Brevibacterium, incorporating the

recombinant DNA in a microorganism belonging to the genus Corynebacterium or Brevibacterium, culturing the microorganism in a medium, and recovering L-threonine or L-isoleucine accumulated in the culture broth. Thus, the present invention relates to the bioindustrial field, and particularly to the field of producing L-threonine and L-isoleucine useful in the industries of medicine, food, and animal feed.

Brief Description of the Drawings

Fig. 1 shows a cleavage map of pChoml for restriction enzymes SalI, PstI, EcoRI and BamHI and the steps for constructing pChoml. The molecular size of plasmids is given in kilobases (Kb). Both HD and HK genes are contained in the chromosome DNA fragment of pChoml indicated by the heavy line.

Fig. 2 shows a cleavage map of the SalI fragment of 3.6 Kb cloned on pChoml for typical restriction enzymes.

Detailed Description of the Invention

According to the present invention, L-threonine and L-isoleucine can be produced in high yield by culturing in a medium a microorganism belonging to the genus Corynebacterium or Brevibacterium and containing a recombinant DNA of a DNA fragment that contains both HD and HK genes of a microorganism belonging to the genus Corynebacterium or Brevibactrium, and a vector DNA, accumulating L-threonine or L-isoleucine in the culture broth, and recovering L-threonine or L-isoleucine therefrom. As for the strain of the genus Corynebacterium or Brevibacterium to be used as a host microorganism, all the microorganisms known as the coryneform glutamic acid-producing bacteria can be used, and preferably the following strains can be used:

Corynebacterium glutamicum	ATCC 31833
Corynebacterium glutamicum	ATCC 13032
Corynebacterium acetoacidophilum	ATCC 13870
Corynebacterium herculis	ATCC 13868
Corynebacterium lilium	ATCC 15990
Brevibacterium divaricatum	ATCC 14020
Brevibacterium flavum	ATCC 14067
Brevibacterium immariophilium	ATCC 14068
Brevibacterium lactofermentum	ATCC 13869
Brevibacterium thiogenitalis	ATCC 19240

As the host microorganism, L-threonine- or L-isoleucine-non-producing strains can be used, but preferably L-threonine-, L-isoleucine- or L-lysine-producing strains are used. The L-threonine-, L-isoleucine- or L-lysine-producing strains can be constructed by deriving mutants which carry amino acid-requiring mutation, amino acid analog-resistant mutation, or a combination of these mutations (PRESCOTT AND DUNN'S INDUSTRIAL MICROBIOLOGY 4th ed. compiled by G. Reed, The AVI Publishing Company Inc. Conn. 1982, pp748 - 801, K. Nakayama).

In the present invention, any microorganism can be used as a supply source of both HD and HK genes, so long as it is a coryneform glutamic acid-producing microorganism having HD and HK activities. For example, wild-type strains belonging to the genus Corynebacterium or Brevibacterium, or L-lysine-, L-threonine- or L-isoleucine-producing mutant strains derived therefrom can be used. The chromosome DNA of these strains can be isolated by subjecting the cells treated with penicillin during the culturing to lysis with lysozyme and a surfactant, removing proteins according to the ordinary procedure and precipitating the DNA with ethanol as disclosed in Japanese Unexamined Published Patent Application No. 126789/83 by the present inventors.

The vector for recombination of a DNA fragment containing both HD and HK genes from the chromosome DNA is not

particularly restricted, so long as it is autonomously replicable in a microorganism belonging to the genus Corynebacterium or Brevibacterium. For example, the plasmids developed by the present inventors, pCG1 (Japanese Unexamined Published Patent Application No. 134500/82), pCG2 (Japanese Unexamined Published Patent Application No. 35197/83), pCG4 and pCG11 (both in Japanese Unexamined Published Patent Application No. 183799/82), pCE54 and pCB101 (both in Japanese Unexamined Published Patent Application No. 105999/83), pCE51 (Japanese Unexamined Published Patent Application No. 34197/85), pCE52 and pCE53 [both in Molecular and General Genetics 196, 175 (1984)], etc. can be used. The plasmid vectors can be isolated and purified as CCC-DNA by subjecting cells to lysis with lysozyme and a surfactant, preparing the cleared lyzates, and precipitating a DNA with polyethyleneglycol, followed by cesium chloride-ethidium bromide density-gradient centrifugation, as disclosed by the present inventors in Japanese Unexamined Published Patent Application Nos. 134500/82 and 186489/82.

A recombinant of a DNA fragment containing both HD and HK genes and a vector plasmid can be produced together with a mixture of various recombinants according to the ordinary procedures [Methods in Enzymology, 68 (1979)], by cleaving the chromosome DNA and the vector plasmid with a restriction enzyme, followed by, if necessary, treatment of the cleaved terminals with a terminal transferase or DNA polymerase, and by ligating both DNAs with a DNA ligase.

A recombinant plasmid containing both HD and HK genes can be obtained by transforming a homoserine (or methionine and threonine)-requiring, HD-deficient mutant strain or a threonine-requiring and homoserine-secreting, HK-deficient mutant strain, derived from a strain of the genus Corynebacterium or Brevibacterium according to ordinary mutation procedures using the said mixture of recombinants, and by selecting a homoserine or threonine-non-requiring

transformant. Transformation of a strain of the genus Corynebacterium or Brevibacterium can be carried out according to a method using protoplasts developed by the present inventors (Japanese Unexamined Published Patent Application Nos. 186492/82 and 186489/82, specifically refer to Examples). The recombinant plasmid containing both HD and HK genes is identified by the ability of complementing the deficiency in HD and HK by retransformation with the thus obtained recombinant plasmid DNA.

When the chromosome DNA of a wild-type strain of the genus Corynebacterium or Brevibacterium is used as a supply source, a recombinant containing both HD and HK genes of wild type is obtained. The productivity of L-threonine and L-isoleucine can be improved by incorporating the recombinant plasmid into a strain of the genus Corynebacterium or Brevibacterium. It is known that in the strains of the genus Corynebacterium or Brevibacterium the HD responsible for the biosynthesis of threonine undergoes feedback inhibition by threonine, and controls the threonine synthesis [Agricultural Biological Chemistry 38 (5), 993 (1974)], and thus the productivity of L-threonine and L-isoleucine can be further improved by using a recombinant plasmid containing a gene coding for the mutant HD relieved from inhibition by threonine.

The recombinant plasmid containing the mutant HD gene can be obtained in the same manner as in the case of the gene of wild-type by isolating a mutant strain resistant to a threonine analog, e.g. α-amino-β-hydroxyvaleric acid (referred to as AHV hereinafter), HD activity of which is relieved from the inhibition by threonine as described in Agricultural Biological Chemistry, 38 (5), 993 (1974), and by using its chromosome DNA as a supply source. Alternatively, the recombinant plasmid containing the gene coding for HD freed from the inhibition by threonine can be obtained by subjecting a strain of the genus Corynebacterium or Brevibacterium

carrying a recombinant plasmid that contains wild-type HD gene to mutation treatment according to the ordinary procedure, whereby endowing the strain with a resistance to a threonine analog.

The recombinant plasmid containing both HD and HK genes of wild-type or mutant-type can be introduced into a microorganism of the genus Corynebacterium or Brevibacterium by said transformation using protoplasts. Production of L-threonine or L-isoleucine by the strains containing the recombinant plasmid is carried out according to a conventional culturing process for producing L-threonine or L-isoleucine by fermentation. That is, by culturing the transformant in an ordinary medium containing a carbon source, a nitrogen source, inorganic compounds, amino acids, vitamins, etc. under aerobic conditions while adjusting the temperature, pH, etc., L-threonine or L-isoleucine is accumulated in the medium during the culturing, and recovered therefrom.

As the carbon source, carbohydrates such as glucose, glycerol, fructose, sucrose, maltose, mannose, starch, starch hydrolyzate, molasses, etc.; polyalcohols; and various organic acids such as pyruvic acid, fumaric acid, lactic acid, acetic acid, etc. can be used. Furthermore, hydrocarbons, alcohols, etc. can be used depending upon the assimilability of the microorganism to be used. Particularly, cane molasses is preferably used.

As the nitrogen source, ammonia; various inorganic and organic ammonium salts such as ammonium chloride, ammonium sulfate, ammonium carbonate, ammonium acetate, etc.; urea and other nitrogen-containing materials as well as various nitrogen-containing organic materials such as peptone, NZ-amine, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, fish meal or its digested product, chrysalis hydrolyzate, etc. can be used.

As the inorganic materials, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium sulfate,

ammonium chloride, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate, etc. can be used. If the vitamins, the amino acids, etc. required for the growth of the microorganism can be supplied to the medium from other medium components described above, it is not necessary to add them to the medium.

Culturing is carried out under aerobic conditions, for example, by shaking culture or by aeration-stirring culture. Generally, preferable culturing temperature is 20 to 40°C. The pH of the medium is preferably maintained around neutrality. Usually, by culturing for 1 to 5 days, L-threonine and/or L-isoleucine is accumulated in the medium. After the culturing, the cells are removed from the culture liquor, and then L-threonine and/or L-isoleucine is recovered from the culture liquor according to a known procedure, for example, by activated carbon treatment or by ion exchange resin treatment.

Thus, L-threonine and/or L-isoleucine can be produced in high yield by using a microorganism of the genus Corynebacterium or Brevibacterium containing a recombinant plasmid that contains both HD and HK genes of a microorganism belonging to the genus Corynebacterium or Brevibacterium.

An example of the present invention is given below:

Example

(1) Preparation of chromosome DNA of Corynebacterium glutamicum ATCC 31833 and vector pCE54:

A seed culture of Corynebacterium glutamicum ATCC 31833 grown in NB medium (a medium containing 20g of bouillon powder and 5g of yeast extract in 1ℓ of deionized water, and adjusted to pH 7.2) was inoculated into 400 mℓ of semi-synthetic medium SSM [a medium containing 20g of glucose, 10g of $(NH_4)_2SO_4$, 3g of urea, 1g of yeast extract, 1g of KH_2PO_4, 0.4g of $MgCl_2 \cdot 6H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 0.2 mg of $MnSO_4 \cdot 4$-$6H_2O$, 0.9 mg of $ZnSO_4 \cdot 7H_2O$, 0.4 mg of $CuSO_4 \cdot 5H_2O$, 0.09 mg of

$Na_2B_4O_7 \cdot 10H_2O$, 0.04 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 30 µg of biotin and 1 mg of thiamine hydrochloride in 1ℓ of water, and adjusted to pH 7.2] and was subjected to shaking culture at 30°C. The optical density at 660 nm (OD) was determined with Tokyo Koden colorimeter, and when OD reached 0.2, penicillin G was added to a final concentration of 0.5 unit/mℓ. Culturing was continued until OD reached 0.6.

Then, the cells were collected from the culture broth and washed with TES buffer solution [0.03M Tris(hydroxymethyl) amino-methane (referred to as Tris hereinafter)-HCl, 0.005M EDTA (disodium ethylenediaminetetraacetate), and 0.05M NaCl, pH 8.0]. The washed cells were suspended in 10 mℓ of a lysozyme solution (25% sucrose, 0.1M NaCl, 0.05M Tris, and 0.8 mg/mℓ lysozyme, pH 8.0; the same solution is used hereinafter as lysozyme solution), and subjected to reaction at 37°C for 4 hours. High molecular chromosome DNA was isolated from the collected cells according to the method of Saito, et al. [Saito, H. et al.: Biochim. Biophys. Acta, 72, 619 (1963)].

pCE54 (Japanese Unexamined Published Patent Application No. 105999/83) used as the vector is a plasmid prepared by linking plasmid pCG2 of Corynebacterium glutamicum (Japanese Unexamined Published Patent Application No. 35197/83 field by the present inventors) with plasmid pGA22 of Escherichia coli [J. Bacteriol. 140, 400 (1979)]. More particularly, it is prepared by linking pCG2 with pGA22 by integration at their single PstI cleavage sites (see Fig. 1). pCE54 was isolated from the cultured cells of Corynebacterium glutamicum ATCC 39019 (a strain having a lysozyme-sensitive mutation and derived from Corynebacterium glutamicum ATCC 31833) carrying pCE54 in the following manner.

The cells were grown till OD reached about 0.7 by shaking culture at 30°C in 400 mℓ of NB medium and were collected. After washing with TES buffer solution, the cells were suspended in 10 mℓ of a lysozyme solution and subjected

to reaction at 37°C for 2 hours. Then, 2.4 mℓ of 5M NaCl, 0.6 mℓ of 0.5M EDTA (pH 8.5), and 4.4 mℓ of a solution containing 4% sodium laurylsulfate and 0.7M NaCl were successively added to the reaction solution, and the thus obtained mixture was gently mixed and placed on ice water for 15 hours. The lysate was transferred into a centrifuge tube and subjected to centrifugation at 69,400 x g at 4°C for 60 minutes to recover a supernatant. Then, polyethyleneglycol (PEG) 6,000 (made by Nakarai Kagaku Yakuhin Co.) in an amount corresponding to 10% by weight was added thereto, and the thus obtained mixture was gently mixed. The solution was placed on ice water, and after 10 hours, subjected to centrifugation at 1,500 x g for 10 minutes to recover pellets. Then, 5 mℓ of TES buffer solution was added to gently redissolve the pellets, and 2.0 mℓ of 1.5 mg/mℓ ethidium bromide was added thereto. Cesium chloride was added and gently dissolved to adjust the density of the solution to 1.580.

The solution was subjected to ultracentrifugation at 105,000 x g at 18°C for 48 hours, and a band at a high density level at the lower position of the centrifuge tube detected under ultraviolet irradiation was withdrawn at the side of the centrifuge tube with a syringe to separate the pCE54 plasmid DNA. The thus obtained fraction was treated 5 times with an equal volume of an isopropyl alcohol solution (consisting of 90% by volume isopropyl alcohol and 10% by volume TES buffer solution and containing cesium bromide at the saturated concentration) to remove ethidium bromide by extraction, and then dialyzed against TES buffer solution.

(2) Cloning of a DNA fragment containing HD and HK genes:

First, 6 units of SalI (made by Takara Shuzo Co.) was added to 60 µℓ of a reaction solution for restriction enzyme SalI (10 mM Tris-HCl, 6 mM $MgCl_2$ and 200 mM NaCl, pH 7.5) containing 3 µg of pCE54 plasmid DNA prepared above, and the mixture was subjected to reaction at 37°C for 60 minutes, and then heated at 65°C for 10 minutes to stop the reaction.

Separately, 4 units of SalI was added to 140 µℓ of the reaction solution for SalI containing 8 µg of chromosome DNA of Corynebacterium glutamicum ATCC 31833, and the mixture was subjected to reaction at 37°C for 60 minutes, and then heated at 65°C for 10 minutes to stop the reaction.

Both reaction mixtures were mixed, and 40 µℓ of a buffer solution for T4 ligase with a 10-fold concentration (660 mM Tris-HCl, 66 mM $MgCl_2$, and 100 mM dithiothreitol, pH 7.6), 40 µℓ of 5 mM ATP, 0.3 µℓ of T4 ligase (1 unit/µℓ, made by Takara Shuzo Co.) and 120 µℓ of purified water were added thereto. The mixture was subjected to reaction at 12°C for 16 hours.

The ligase reaction mixture was used to transform K53 strain derived from a lysozyme-sensitive mutant strain originating from Corynebacterium glutamicum ATCC 31833 [K53 strain carries homoserine-requiring mutation (HD defect) and leucine-requiring mutation]. K53 strain was deposited as FERM P-8257 with the Fermentation Research Institute (FRI), Agency of Industrial Science and Technology on May 23, 1985 and transferred to the deposit under the Budapest Treaty as FERM BP-1052 on May 19, 1986.

For the transformation, protoplasts prepared in the following manner were used.

A seed culture of K53 strain was inoculated in NB medium and subjected to shaking culture at 30°C, and the cells were collected when OD reached 0.6. The cells were suspended in a solution (pH 7.6) containing 1 mg/mℓ lysozyme in RCGP medium [5g of glucose, 5g of casamino acid, 2.5g of yeast extract, 3.5g of K_2HPO_4, 1.5g of KH_2PO_4, 0.41g of $MgCl_2 \cdot 6H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 2 mg of $MnSO_4 \cdot 4\text{-}6H_2O$, 0.9 mg of $ZnSO_4 \cdot 7H_2O$, 0.04 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 30 µg of biotin, 2 mg of thiamine hydrochloride, 135g of disodium succinate, and 30g of polyvinylpyrrolidone (molecular weight: 10,000) in 1ℓ of water] to make about 10^9 cells/mℓ, and the suspension was transferred into an L-tube and subjected to gentle shaking culture at 30°C for 5 hours to make protoplasts.

Then, 0.5 mℓ of the protoplast suspension was taken into a small test tube, and subjected to centrifugation at 2,500 x g for 5 minutes. The protoplasts were suspended again in 1 mℓ of TSMC buffer solution (10 mM $MgCl_2$, 30 mM $CaCl_2$, 50 mM Tris-HCl, and 400 mM sucrose, pH 7.5), subjected to centrifugation, and then suspended in 0.1 mℓ of TSMC buffer solution. Then, 100 µℓ of a 1:1 mixture of TSMC buffer solution at a two-fold concentration and the said ligase reaction solution was added to the cell suspension, and 0.8 mℓ of TSMC buffer solution containing 20% PEG 6,000 was added thereto. After 3 minutes, 2 mℓ of RCGP medium (pH 7.2) was added thereto, and the mixture was subjected to centrifugation at 2,500 x g for 5 minutes to remove the supernatant. The precipitated protoplasts were suspended in 1 mℓ of RCGP medium, and then 0.2 mℓ of the suspension was spread onto RCGP agar medium (a medium containing 1.4% agar in RCGP medium, pH 7.2) containing 300 µg/mℓ kanamycin, and cultured at 30°C for 7 days.

Colonies grown on the agar medium were collected. The cells were centrifuged, washed twice with physiological saline solution, and then suspended in 1 mℓ of physiological saline solution. Then, the cell suspension was spread again on minimal agar medium M1 [a medium containing 10g of glucose, 1g of $NH_4H_2PO_4$, 0.2g of KCl, 0.2g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 0.2 mg of $MnSO_4 \cdot 4\text{-}6H_2O$, 0.9 mg of $ZnSO_4 \cdot 7H_2O$, 0.4 mg of $CuSO_4 \cdot 5H_2O$, 0.09 mg of $Na_2B_4O_7 \cdot 10H_2O$, 0.04 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 µg of biotin, 2.5 mg of p-aminobenzoic acid, 1 mg of thiamine hydrochloride, and 16g of agar in 1ℓ, and adjusted to pH 7.2] containing 50 µg/mℓ leucine and 20 µg/mℓ kanamycin, and cultured at 30°C for 3 days, and transformants having a homoserine non-requirement and a kanamycin resistance were selected.

The transformants were cultured in NB medium, and plasmid DNAs were isolated from the cells by the same method as used for isolating pCE54 in said step (1).

It was found as a result of analysis by digestion with various restriction enzymes and agarose gel electrophoresis that the plasmid obtained from one of the transformants and named "pChoml" was a plasmid wherein a SalI DNA fragment of 3.6 Kb was inserted at the single SalI cleavage site of pCE54. The SalI DNA fragment had two PstI cleavage sites and one EcoRI cleavage site at the positions shown in Fig. 1.

The transformant of K53 strain selected by kanamycin resistance after retransformation with pChoml DNA in the same manner as above showed homoserine non-requirement at the same time, and the plasmid isolated therefrom had the same structure as that of pChoml. These results support that the HD gene of Corynebacterium glutamicum ATCC 31833 was cloned onto pChoml.

The presence of HK gene on pChoml was confirmed in the following manner.

Protoplasts of threonine-requiring, homoserine-producing, HK-deficient mutant strain K54 derived from Corynebacterium glutamicum ATCC 31833 and deposited as FERM P-8258 with the FRI on May 23, 1985 (transferred to the deposit under the Budapest Treaty as FERM BP-1053 on May 19, 1986) were transformed with pChoml DNA.

The protoplasts of the strain were prepared from penicillin-treated cells in the following manner.

First, 0.1 mℓ of a seed culture obtained using NB medium was inoculated in 10 mℓ of SSM medium containing 100 µg/mℓ threonine, and subjected to shaking culture at 30°C. Penicillin G was added to a final concentration of 0.45 unit/mℓ when OD reached 0.15. Culturing was further continued, and when OD reached 0.6, the cells were collected and treated with lysozyme by the same method as used above for preparing the protoplast of K53 strain to make a protoplast. Transformation was also conducted in the same manner as above, and a transformant was selected on RCGP agar medium containing

300 µg/mℓ kanamycin. The kanamycin-resistant transformant was threonine non-requiring at the same time.

The transformant was subjected to shaking culture in 400 mℓ of SSM medium, and penicillin G was added to make 0.5 unit/mℓ when OD reached 0.2. Culturing was further continued till OD reached about 0.6, and the cells were collected. The collected cells were subjected to lysis in the same manner as described in step (1), and a plasmid was isolated from the lysate by cesium chloride-ethidium bromide density gradient centrifugation. As a result of analysis by agarose gel electrophoresis after digestion with various restriction enzymes, it was confirmed that the plasmid was the same as pChoml.

It was found from the foregoing that the SalI DNA fragment of 3.6 Kb cloned on pChoml had both HD and HK genes.

A cleavage map of the SalI fragment of 3.6 Kb cloned on pChoml for typical restriction enzymes is illustrated in Fig. 2.

(3) Isolation of a mutant plasmid endowing a host microorganism with a high AHV resistance:

K53 strain carrying pChoml was grown in NB medium containing 25 µg/mℓ kanamycin up to the late stage of logarithmic growth phase. Cells were centrifuged twice with 50 mM Tris-maleic acid buffer solution (pH 6.0), suspended in 50 mM Tris-maleic acid buffer solution (pH 6.0) containing 400 µg/mℓ N-methyl-N'-nitro-N-nitrosoguanidine, and treated at room temperature for 30 minutes. The treated cells were centrifuged and washed with the same buffer solution as used above twice. The cells were suspended in NB medium and subjected to shaking culture at 30°C for 2 hours. The cultured cells were centrifuged and washed twice with physiological saline solution, and suspended in physiological saline solution. The cell suspension was spread onto minimal agar medium M1 containing 20 µg/mℓ kanamycin and 6 mg/mℓ AHV,

and cultured at 30°C for 3 days. One of the colonies formed was purified, and a plasmid was isolated from the cultured cells in the same manner as above. The plasmid was named pChom10.

Cultured cells of K53 strains containing pChom1 and pChom10, respectively, were centrifuged and washed with physiological saline solution, and the microorganisms in an amount corresponding to about 10^4 cells were spread onto minimal agar media M1 containing 2 mg/mℓ, 4 mg/mℓ and 6 mg/mℓ AHV, respectively, to make comparison of the degree of AHV resistance between these two strains. As a result of culturing at 30°C for 3 days, the strain carrying pChom1 grew on M1 agar medium containing 2 mg/mℓ AHV, but failed to grow in the agar medium containing 4 mg/mℓ AHV. On the other hand, the strain containing pChom10 grew even on M1 agar medium containing 6 mg/mℓ AHV.

As a result of cleavage analysis with various restriction enzymes, it was found that pChom10 had the same structure as that of pChom1, and also had a complementarity of threonine-requiring, HK-deficient mutant strain K54.

(4) Production of threonine by the strain containing pChom1 or pChom10:

Corynebacterium glutamicum ATCC 31833, Corynebacterium herculis ATCC 13868, Brevibacterium lactofermentum ATCC 13869 and a lysine-producing strain Brevibacterium flavum ATCC 21475 [resistant to S-(2-aminoethyl)cysteine] were transformed with pChom1 and pChom10. Protoplasts were prepared in the same manner as in the preparation of the protoplasts of K54 strain in step (2). That is, the protoplasts were prepared by treating the cells with penicillin G (0.45 unit/mℓ) in the course of culturing in SSM medium, and then by treating the cultured cells with lysozyme.

The protoplasts were transformed with 1 µg of the plasmid DNA in the same manner as above, and kanamycin-

resistant transformants were selected on RCGP agar medium. The plasmids were isolated from the transformants in the same manner as in the isolation of the plasmid from the pChom1-transformed strain of K54 strain in step (2), and it was confirmed by cleavage analysis with various restriction enzymes that each transformant carried pChom1 or pChom10.

Threonine production tests on the transformants and their respective parent strains were carried out in the following manner.

First, 0.5 mℓ of a seed culture obtained by shaking culture in NB medium at 30°C for 16 hours was inoculated in a test tube containing 5 mℓ of a production medium [a medium containing 100g of glucose, 20g of $(NH_4)_2SO_4$, 0.5g of KH_2PO_4, 0.5g of K_2HPO_4, 1g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4\text{-}6H_2O$, 100 µg of biotin, and 20g of calcium carbonate in 1ℓ of water, and adjusted to pH 7.2], and subjected to shaking culture at 30°C for 72 hours. After the culturing, the culture filtrate was subjected to paper chromatography, and the amount of the produced L-threonine was determined by colorimetry using ninhydrin coloration after paper chromatography. The results are shown in Table 1.

Table 1

Strain	Amount of the threonine produced (g/ℓ)
Corynebacterium glutamicum ATCC31833	0
" ATCC31833/pChom1	0.4
" ATCC31833/pChom10	1.9
Corynebacterium herculis ATCC13868	0
" ATCC13868/pChom1	0.6
" ATCC13868/pChom10	2.2
Brevibacterium lactofermentum ATCC13869	0
" ATCC13869/pChom1	0.3
" ATCC13869/pChom10	1.7
Brevibacterium flavum ATCC21475	0
" ATCC21475/pChom1	0.4
" ATCC21475/pChom10	5.2

(5) Production of isoleucine by the strain containing pChom1 or pChom10:

Corynebacterium glutamicum FERM P-7160 (FERM BP-455), Brevibacterium flavum ATCC 14067 and a lysine-producing strain Corynebacterium glutamicum FERM BP-158 were transformed in the same manner as in (4) above to obtain transformants carrying pChom1 and pChom10. It was confirmed in the same manner as above that the transformants had the plasmids. Isoleucine production tests on the parent strains and the transformants were carried out under the same conditions as in (4). The results are shown in Table 2.

Table 2

Strain	Amount of the isoleucine produced (g/ℓ)
Corynebacterium glutamicum FERM P-7160	1.2
" FERM P-7160/pChom1	2.6
" FERM P-7160/pChom10	5.3
Brevibacterium flavum ATCC14067	0
" ATCC14067/pChom1	0.6
" ATCC14067/pChom10	3.7
Corynebacterium glutamicum FERM BP-158	0
" FERM BP-158/pChom1	0.9
" FERM BP-158/pChom10	4.8

(6) Subcloning of HD and HK genes:

The recombinant plasmid containing the fragment between the SmaI cleavage site and the right cleavage site among the three PvuII cleavage sites illustrated in Fig. 2 as dark area was obtained in the following manner.

First, 3 units of EcoRI (product of Takara Shuzo Co.) was added to 20 µℓ of a reaction solution for EcoRI (100 mM Tris-HCl, 6 mM $MgCl_2$, and 50 mM NaCl, pH 7.5) containing one µg of pCE54 plasmid DNA. Reaction was carried out at 37°C for 60 minutes and stopped by heating at 70°C for 15 minutes. Deoxy ATP and deoxy TTP (0.05 mM each) were added, and then 3 units of Escherichia coli DNA polymerase I large fragment (product of Takara Shuzo Co.) was added. Reaction was carried out at 37°C for 30 minutes and stopped by heating at 70°C for 15 minutes.

Separately, 3 units of SmaI (product of Takara Shuzo Co.) and one unit of PvuII (product of Takara Shuzo Co.) were added to 20 µℓ of a reaction solution for SmaI (10 mM Tris-

HCl, 20 mM KCl, and 6 mM $MgCl_2$, pH 7.5) containing 3 µg of pChoml plasmid DNA, and reaction was carried out at 37°C for 60 minutes. A DNA fragment of 2.6 Kb was obtained by fractionation and purification from the reaction product using the method described in Molecular Cloning (Cold Spring Harbor Laboratory, 1982) p.164. That is, the reaction product was subjected to agarose gel electrophoresis to cut off a gel band of 2.6 Kb, and the gel band was subjected to electrophoresis in a dialysis membrane to extract the DNA fragment. Three-fold volume of ethanol was added to the extract. The mixture was cooled at -80°C for 10 minutes and a precipitate was recovered by centrifugation. After evaporating ethanol in vacuo, the residue was dissolved in 20 µℓ of the reaction solution for EcoRI.

Both reaction products were mixed, and 5 µℓ of a buffer solution for T4 ligase with a 10-fold concentration, 5 µℓ of 5 mM ATP and one unit of T4 ligase (product of Takara Shuzo Co.) were added. Reaction was carried out at 12°C for 16 hours. Protoplasts of K53 strain were prepared by the method described above and transformation was carried out using the ligase reaction product. A plasmid DNA was prepared from one of the kanamycin-resistant and homoserine-non-requiring transformants by the method described above and was named pChom20.

pChom20 was cleaved with PstI and analyzed by agarose gel electrophoresis. As the result, it was confirmed that a region of 2.6 Kb containing a PstI fragment of 1.0 Kb of the inserted SalI fragment of 3.6 Kb of pChoml and illustrated in Fig. 1 was subcloned on pChom20.

K53 strain and K54 strain were again transformed with pChom20. As a result of examination, it was confirmed that the plasmid DNA had the ability of complementing both HD and HK defects.

Activities of HD and HK were measured by the method described in J. Biochem. 68, 311 (1970) and ibid. 71, 219

(1972). As the result, the HD activity of the transformant of K54 strain carrying pChom20 was 16 times that of K54 strain, and the HK activity of the transformant of K53 strain carrying pChom20 was 17 times that of K53 strain. These results are the same as in the case of the transformants carrying pChom1. From the foregoing, it was confirmed that HD and HK genes were present on the fragment of 2.6 Kb from SmaI to PvuII.

Three units of HindIII (product of Takara Shuzo Co.) was added to 20 µℓ of a reaction solution for HindIII (10 mM Tris-HCl, 6 mM $MgCl_2$, and 60 mM NaCl, pH 7.5) containing 1 µg of pChom1 DNA. Reaction was carried out at 37°C for 60 minutes and stopped by heating at 70°C for 15 minutes. Deoxy ATP, deoxy GTP, deoxy CTP and deoxy TTP (0.05 mM each) were added and 3 units of Escherichia coli DNA polymerase I large fragment (product of Takara Shuzo Co.) was added. Reaction was carried out at 37°C for 30 minutes and stopped by heating at 70°C for 15 minutes. One µℓ of 2M NaCl was added and 3 units of SalI (product of Takara Shuzo Co.) was added. Reaction was carried out at 37°C for one hour. The reaction product was subjected to agarose gel electrophoresis. A DNA fragment of 2.5 Kb was purified by the method described above and dissolved in 20 µℓ of the reaction solution for HindIII.

Separately, 3 units of EcoRI was added to 20 µℓ of the reaction solution for EcoRI containing 1 µg of pCE54 plasmid DNA. Reaction was carried out at 37°C for 60 minutes and stopped by heating at 70°C for 15 minutes. Deoxy ATP and deoxy TTP (0.05 mM each) were added to the reaction product and further 3 units of Escherichia coli DNA polymerase I large fragment was added. Reaction was carried out at 37°C for 30 minutes and stopped by heating at 70°C for 15 minutes. One µℓ of 2M NaCl was added to the reaction product and further 3 units of SalI was added. Reaction was carried out at 37°C for one hour and the reaction product was subjected to agarose gel electrophoresis. A fragment of 12.5 Kb was purified by the method described above and dissolved in 20 µℓ of the reaction solution for HindIII.

Both solutions of DNA fragments were mixed, and 5 µℓ of a buffer solution for T4 ligase with a 10-fold concentration, 5 µℓ of 5 mM ATP and 1 unit of T4 ligase were added. Reaction was carried out at 12°C for 16 hours. Protoplasts of K54 strain were prepared by the same method as mentioned above and transformation was carried out using the ligase reaction product. A plasmid DNA was prepared from one of the kanamycin-resistant and threonine-non-requiring transformants by the method mentioned above. It was confirmed by the analysis with restriction enzymes that the HindIII-SalI fragment of 2.5 Kb in the SalI fragment of 3.6 Kb was subcloned in the plasmid DNA named pChom21.

HD and HK activities of K54 strain carrying pChom20 or pChom21 were measured by the method described in J. Biochem. 68, 311 (1970) and ibid. 71, 219 (1972). As the result, it was found that the HD activity of pChom21-carrying strain was less than 1/15 of that of pChom20-carrying strain and the HK activity was more than 5/6. It was recognized from the fact that the region upstream from HindIII cleavage site present in the region of 75 base pairs upstream from the initiation codon of HD is required for complete expression of HD.

(7) Nucleotide sequence of the DNA fragment containing both HD and HK genes:

Whole nucleotide sequence of the DNA fragment containing both HD and HK genes subcloned in pChom1 and pChom20 was determined using the method described in Methods in Enzymology 101, 20, 1983. That is, after a restriction cleavage map was prepared by a conventional method, a single stranded DNA was prepared by subcloning into M13 phage vector and the nucleotide sequence was determined by chain termination method using dideoxynucleotide.

The result is illustrated in Table 3. Table 3 shows the nucleotide sequence of the SmaI-PvuII DNA fragment

consisting of 2615 base pairs and the amino acid sequence corresponding to two open reading frames (from base No. 322 to base No. 1557 and from base No. 1571 to base No. 2497) present in the DNA fragment. These open reading frames correspond to HD and HK structural genes, respectively.

ATCC 39019 strain containing pCE54 and ATCC 31833 strain have been deposited with the ATCC under the Budapest Treaty.

Table 3

```
                                                                    60
CCCGGGTTGATATTAGATTTCATAAATATACTAAAAATCTTGAGAGTTTTTCCGGTTGAA

                                                                   120
AACTAAAAAGCTGGGAAGGTGAATCGAATTTCGGGGCTTTAAAGCAAAAATGAACAGCTT

                                                                   180
GGTCTATAGTGGCTAGGTACCCTTTTTGTTTTGGACACATGTAGGGTGGCCGAAACAAAG

                                                                   240
TAATAGGACAACAACGCTCGACCGCGATTATTTTTGGAGAATCATGACCTCAGCATCTGC

                                                                   300
CCCAAGCTTTAACCCCGGCAAGGTCCCGGCTCAGCAGTCGGAATTGCCCTTTTAGGATTC

                                                                   360
GGAACAGTCGGCACTGAGGTGATGCGTCTGATGACCGAGTACGGTGATGAACTTGCGCAC
                                  MetArgLeuMetThrGluTyrGlyAspGluLeuAlaHis

                                                                   420
CGCATTGGTGGCCCACTGGAGGTTCGTGGCATTGCTGTTTCTGATATCTCAAAGCCACGT
ArgIleGlyGlyProLeuGluValArgGlyIleAlaValSerAspIleSerLysProArg

                                                                   480
GAAGGCGTTGCACCTGAGCTGCTCACTGAGGACGCTTTTGCACTCATCGAGCGCGAGGAT
GluGlyValAlaProGluLeuLeuThrGluAspAlaPheAlaLeuIleGluArgGluAsp

                                                                   540
GTTGACATCGTCGTTGAGGTTATCGGCGGCATTGAGTACCCACGTGAGGTAGTTCTCGCA
ValAspIleValValGluValIleGlyGlyIleGluTyrProArgGluValValLeuAla

                                                                   600
GCTCTGAAGGCCGGCAAGTCTGTTGTTACCGCCAATAAGGCTCTTGTTGCAGATCACTCT
AlaLeuLysAlaGlyLysSerValValThrAlaAsnLysAlaLeuValAlaAspHisSer

                                                                   660
GCTGAGCTTGCTGATGCAGCGGAAGCCGCAAACGTTGACCTGTACTTCGAGGCTGCTGTT
AlaGluLeuAlaAspAlaAlaGluAlaAlaAsnValAspLeuTyrPheGluAlaAlaVal

                                                                   720
GCAGGCGCAATTCCAGTGGTTGGCCCACTGCGTCGCTCCCTGGCTGGCGATCAGATCCAG
AlaGlyAlaIleProValValGlyProLeuArgArgSerLeuAlaGlyAspGlnIleGln

                                                                   780
TCTGTGATGGGCATCGTTAACGGCACCACCAACTTCATCTTGGACGCCATGGATTCCACC
SerValMetGlyIleValAsnGlyThrThrAsnPheIleLeuAspAlaMetAspSerThr

                                                                   840
GGCGCTGACTATGCAGATTCTTTGGCTGAGGCAACTCGTTTGGGTTACGCCGAAGCTGAT
GlyAlaAspTyrAlaAspSerLeuAlaGluAlaThrArgLeuGlyTyrAlaGluAlaAsp

                                                                   900
CCAACTGCAGACGTCGAAGGCCATGACGCCGCATCCAAGGCTGCAATTTTGGCATCCATC
ProThrAlaAspValGluGlyHisAspAlaAlaSerLysAlaAlaIleLeuAlaSerIle
```

Table 3 (Cont'd)

960
GCTTTCCACACCCGTGTTACCGCGGATGATGTGTACTGCGAAGGTATCAGCAACATCAGC
AlaPheHisThrArgValThrAlaAspAspValTyrCysGluGlyIleSerAsnIleSer

1020
GCTGCCGACATTGAGGCAGCACAGCAGGCAGGCCACACCATCAAGTTGTTGGCCATCTGT
AlaAlaAspIleGluAlaAlaGlnGlnAlaGlyHisThrIleLysLeuLeuAlaIleCys

1080
GAGAAGTTCACCAACAAGGAAGGAAAGTCGGCTATTTCTGCTCGCGTGCACCCGACTCTA
GluLysPheThrAsnLysGluGlyLysSerAlaIleSerAlaArgValHisProThrLeu

1140
TTACCTGTGTCCCACCCACTGGCGTCGGTAAACAAGTCCTTTAATGCAATCTTTGTTGAA
LeuProValSerHisProLeuAlaSerValAsnLysSerPheAsnAlaIlePheValGlu

1200
GCAGAAGCAGCTGGTCGCCTGATGTTCTACGGAAACGGTTGCAGGTGGCGCGCAAACGGT
AlaGluAlaAlaGlyArgLeuMetPheTyrGlyAsnGlyCysArgTrpArgAlaAsnGly

1260
CTGCTGTGCTTGGCGACGTCGTTGGAGCCGCACGAAACAAGGTGCACGGTGGCCGCTGTC
LeuLeuCysLeuAlaThrSerLeuGluProHisGluThrArgCysThrValAlaAlaVal

1320
CAGGTGAGTCCACCTACGCTAACCTGCCGATCGCTGATTTCGGTGAGACCACCACTCGTT
GlnValSerProProThrLeuThrCysArgSerLeuIleSerValArgProProLeuVal

1380
ACCACCTCGACATGGATGTGGAAGATCGCGTGGGCGTTTTGGCTGAATTGGCTAGCCTGT
ThrThrSerThrTrpMetTrpLysIleAlaTrpAlaPheTrpLeuAsnTrpLeuAlaCys

1440
TCTCTGAGCAAGGAATCTCCCTGCGTAACAATCCGACAGGAAGAGCGCGATGATGATGCA
SerLeuSerLysGluSerProCysValThrIleArgGlnGluGluArgAspAspAspAla

1500
CGTCTGATCGTTGTCACCCACTCTGCGCTGGAATCTGATCTTTCCCGCACCGTTGAACTG
ArgLeuIleValValThrHisSerAlaLeuGluSerAspLeuSerArgThrValGluLeu

1560
CTGAAGGCTAAGCCTGTTGTTAAGGCAATCAACAGTGTGATCCGCCTCGAAAGGGACTAA
LeuLysAlaLysProValValLysAlaIleAsnSerValIleArgLeuGluArgAsp***

1620
TTTTACTGACATGGCAATTGAACTGAACGTCGGTCGTAAGGTTACCGTCACGGTACCTGGA
MetAlaIleGluLeuAsnValGlyArgLysValThrValThrValProGly

1680
TCTTCTGCAAACCTCGGACCTGGCTTTGACACTTTAGGTTTGGCACTGTCGATATACGAC
SerSerAlaAsnLeuGlyProGlyPheAspThrLeuGlyLeuAlaLeuSerIleTyrAsp

1740
ACTGTCGAAGTGGAAATTATTCCATCTGGCTTGGAAGTGGAAGTTTTTGGCGAAGGCCAA
ThrValGluValGluIleIleProSerGlyLeuGluValGluValPheGlyGluGlyGln

1800
GGAGAAGTCCCTCTTGATGGCTCCCACCTGGTGGTTAAAGCTATTCGTGCTGGCCTGAAG
GlyGluValProLeuAspGlySerHisLeuValValLysAlaIleArgAlaGlyLeuLys

Table 3 (Cont'd)

```
                                                                    1860
GCAGCTGACGCTGAAGTGCCTGGATTGCGAGTGGTGTGCCACAACAACATTCCGCAGTCT
AlaAlaAspAlaGluValProGlyLeuArgValValCysHisAsnAsnIleProGlnSer

                                                                    1920
CGTGGTCTTGGTTCCTCTGCTGCAGCGGCGGTTGCTGGTGTTGCAGCAGCTAATGGTTTG
ArgGlyLeuGlySerSerAlaAlaAlaAlaValAlaGlyValAlaAlaAlaAsnGlyLeu

                                                                    1980
GCGGATTTTCCGCTGACTCAAGAGCAGATTGTTCAGTTGTCCTCTGCCTTTGAAGGCCAC
AlaAspPheProLeuThrGlnGluGlnIleValGlnLeuSerSerAlaPheGluGlyHis

                                                                    2040
CCAGATAATGCTGCGGCTTCTGTGCTGGGCGGACGAGTGGTGTCGTGGACAAATCTGTCT
ProAspAsnAlaAlaAlaSerValLeuGlyGlyArgValValSerTrpThrAsnLeuSer

                                                                    2100
ATCGACGGCAAGAGCCAGCCACAGTATGCTGCTGTACCACTTGAGGTGCAGGATAATATT
IleAspGlyLysSerGlnProGlnTyrAlaAlaValProLeuGluValGlnAspAsnIle

                                                                    2160
CGTGCGACTGCGCTGGTTCCTAATTTCCACGCATCCACCGAAGCTGTGCGCCGAGTCCTT
ArgAlaThrAlaLeuValProAsnPheHisAlaSerThrGluAlaValArgArgValLeu

                                                                    2220
CCAACTGAAGTCACTCACATCGATGCGCGATTCAACGTGTCCCGCGTTGCGGTGATGATC
ProThrGluValThrHisIleAspAlaArgPheAsnValSerArgValAlaValMetIle

                                                                    2280
GTTGCATTGCAGCAGCGTCCTGATCTGCTGTGGGAGGGTACTCGTGACCGACTGCACCAG
ValAlaLeuGlnGlnArgProAspLeuLeuTrpGluGlyThrArgAspArgLeuHisGln

                                                                    2340
CCTTATCGTGCAGAAGTGTTGCCCGTTACCTCCGAATGGGTAAACCGTCTGCGCAACCGT
ProTyrArgAlaGluValLeuProValThrSerGluTrpValAsnArgLeuArgAsnArg

                                                                    2400
GGCTATGCAGCGTACCTTTCCGGTGCCGGCCCAACCGCCATGGTGCTGTCCACTGAGCCA
GlyTyrAlaAlaTyrLeuSerGlyAlaGlyProThrAlaMetValLeuSerThrGluPro

                                                                    2460
ATTCCAGACAAGGTTTTGGAAGATGCTCGTGAGTCTGGCATTAAGGTGCTTGAGCTTGAG
IleProAspLysValLeuGluAspAlaArgGluSerGlyIleLysValLeuGluLeuGlu

                                                                    2520
GTTGCGGGACCAGTCAAGGTTGAAGTTAACCAACCTTAGGCCCAACAAGGAAGCCCCCTT
ValAlaGlyProValLysValGluValAsnGlnPro***

                                                                    2580
CGAATCAAGAAGGGGGCCTTATTAGTGAGCAATTATTCGCTGAACACGTGAACCTTACA

GGTGCCCGGCGCGTTGAGTGGTTTAGTTCCAGCTG
```

As illustrated in Table 4, similar base sequences exist in the region of 120 base pairs upstream from the initiation codon of the open reading frame of HD gene and that upstream from the initiation codon of the open reading frame of HK gene (the C-terminal region of the open reading frame of HD gene). These sequences are required for the expression of both genes. Further, as indicated with the underline in Table 5, a potential stem and loop structure is detected within 60 base pairs downstream from the termination codon of the open reading frame of HK gene, and is considered as the transcription termination signal of the bacteria belonging to the genus Corynebacterium or Brevibacterium.

In Table 4, the similar sequences of HD and HK are illustrated. The underlined parts are common in both sequences. Amino acid sequence from the initiation codon to the fifth residue is illustrated under the DNA sequences. Stp upstream from the HK initiation codon shows the termination codon of HD.

Table 5 shows the DNA sequence of the C-terminal region of HK gene and the region downstream therefrom.

Table 4

Hind III
↓
HD : CGATTATTTTTGGAGAATCATGACCTCAGCATCTGCCCCAAGCTTTAA

CCCCGGCAAGGTCCCGGC

HK : TCACCCACTCTGCGCTGGAATCTGATCTTTCCCGCACCGTTGAACTGC

TGAAGGCTAAGCCTGTTG

TCAGCAGTCGGAATTGCCCTTTTAGGATTCGGAACAGTCGGCACTGAG

GTGATGCGTCTGATGACC
MetArgLeuMetThr

TTAAGGCAATCAACAGTGTGATCCGCCTCGAAAGGGACTAATTTTACTGA
Stp

CATGGCAATTGAACTG
MetAlaIleGluLeu

Table 5

GTTAACCAACCTTAGGCCCAACAAGGAAGCCCCCTTCGAATCAAGAAGGGGGCCTT
ValAsnGlnProStp

ATTAGTGAGCAA

What is Claimed is:

1. A process for producing L-threonine or L-isoleucine, which comprises culturing in a medium a microorganism belonging to the genus Corynebacterium or Brevibacterium and containing a recombinant DNA of a DNA fragment carrying genetic information responsible for the synthesis of homoserine dehydrogenase and homoserine kinase of a microorganism belonging to the genus Corynebacterium or Brevibacterium and a vector DNA, accumulating L-threonine or L-isoleucine in the culture broth, and recovering L-threonine or L-isoleucine therefrom.

2. A process according to Claim 1, wherein the vector is replicable in a microorganism belonging to the genus Corynebacterium or Brevibacterium and selected from pCG1, pCG2, pCG4, pCG11, pCE51, pCE52, pCE53, pCE54, pCB101 and plasmids derived therefrom.

3. A recombinant DNA which contains a DNA fragment carrying genetic information responsible for the synthesis of homoserine dehydrogenase and homoserine kinase originating from a microorganism belonging to the genus Corynebacterium or Brevibacterium and capable of endowing a host microorganism belonging to the genus Corynebacterium or Brevibacterium with a resistance to an analog of threonine or isoleucine.

4. A microorganism belonging to the genus Corynebacterium or Brevibacterium and containing a recombinant DNA of a DNA fragment carrying genetic information responsible for the synthesis of homoserine dehydrogenase and homoserine kinase originating from a microorganism belonging to the genus Corynebacterium or Brevibacterium and a vector DNA.

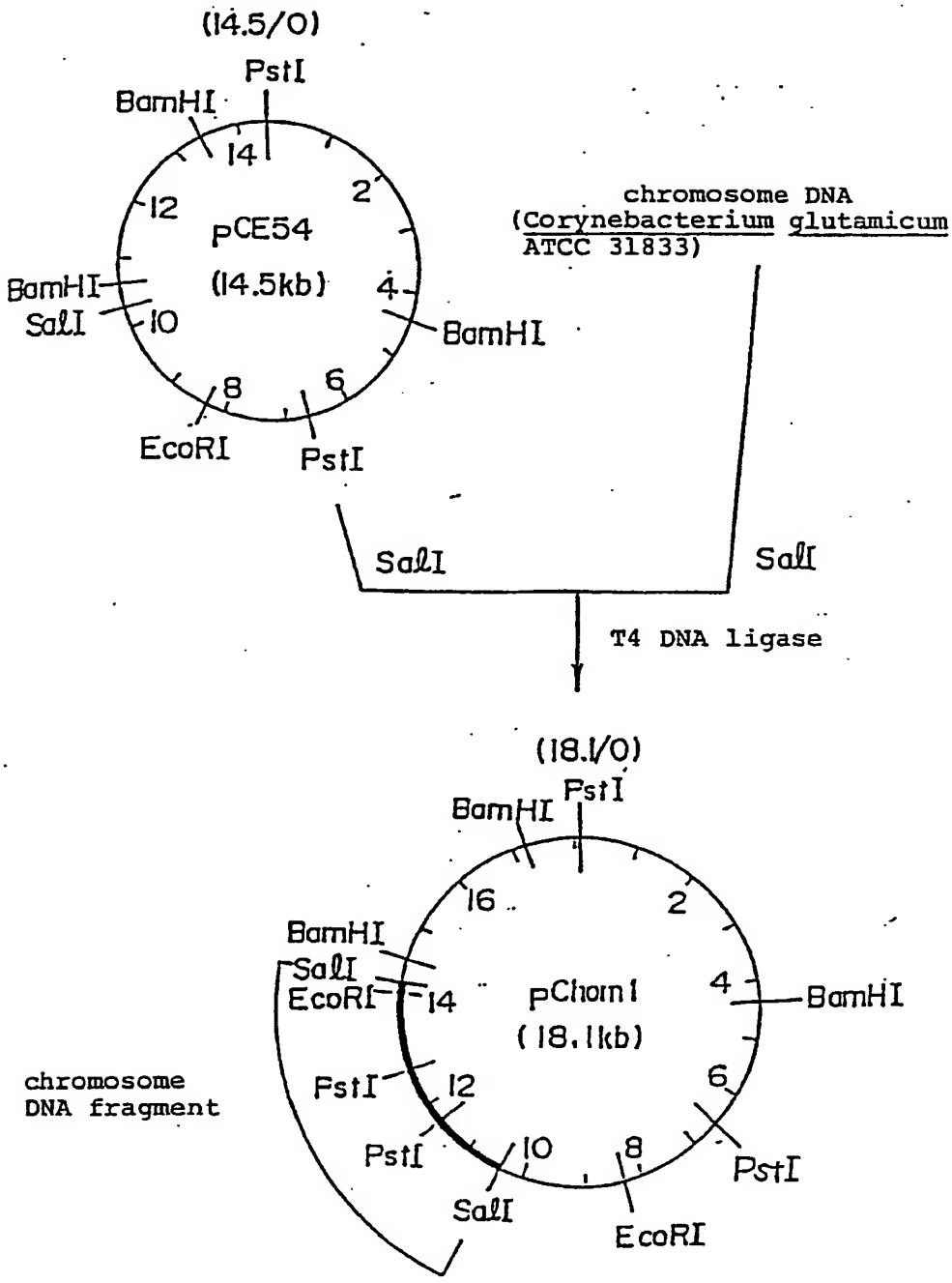
5. A DNA coding for the amino acid sequence of homoserine dehydrogenase as illustrated in Table 3.

6. A DNA coding for the amino acid sequence of homoserine kinase as illustrated in Table 3.

7. A process for producing L-threonine or L-isoleucine, which comprises introducing a recombinant DNA containing a DNA fragment carrying genetic information responsible for the synthesis of homoserine dehydrogenase and homoserine kinase of a microorganism belonging to the genus Corynebacterium or Brevibacterium into a lysine-producing microorganism belonging to the genus Corynebacterium or Brevibacterium, culturing the thus obtained transformant in a medium, accumulating L-threonine or L-isoleucine in the medium and recovering L-threonine or L-isoleucine therefrom.

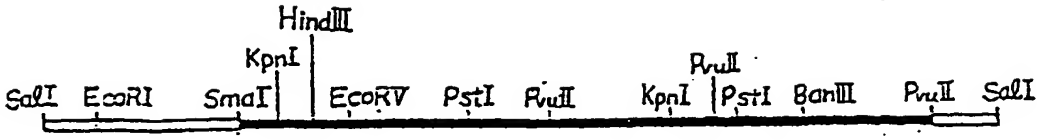
8. A DNA sequence necessary for expressing a foreign gene in a microorganism belonging to the genus Corynebacterium or Brevibacterium, which contains 120 nucleotides sequence upstream from the initiation codon of the open reading frame coding for homoserine dehydrogenase or homoserine kinase or a part thereof and 60 nucleotides sequence downstream from the termination codon of the open reading frame coding for homoserine kinase or a part thereof in the DNA sequence illustrated in Table 3.

Fig. 1


(14.5/0)
PstI
BamHI
14
2
12
pCE54
(14.5kb)
BamHI
4
SalI
10
BamHI
8
6
EcoRI
PstI
chromosome DNA
(Corynebacterium glutamicum
ATCC 31833)
SalI
SalI
T4 DNA ligase
(18.1/0)
PstI
BamHI
16
2
BamHI
SalI
EcoRI
14
pChorn1
(18.1kb)
4
BamHI
PstI
12
6
PstI
10
8
PstI
SalI
EcoRI
chromosome
DNA fragment

2/2

Fig. 2


HindIII
KpnI
PvuII
SalI
EcoRI
SmaI
EcoRV
PstI
PvuII
KpnI
PstI
BanIII
PvuII
SalI

European Patent Office

Application number:
0204326

DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits: ATTC 39019
ATTC 31833